The *in vitro* Hydrolysis of Phytosterol Conjugates in Food Matrices by Mammalian Digestive Enzymes

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ABSTRACT: All fruits, vegetables, and grains contain phytosterols. Numerous clinical studies have documented that phytosterols lower LDL-cholesterol levels and thereby reduce the risk of cardiovascular disease. Most experts believe that the cholesterol-lowering mechanism of phytosterols requires that they be in their "free" form. In addition to their occurrence in the free form, phytosterols also occur as four common phytosterol conjugates: (i) fatty acyl esters, (ii) hydroxycinnamate esters, (iii) steryl glycosides, and (iv) fatty acylated steryl glycosides. This study was undertaken to investigate the extent of hydrolysis of four common phytosterol conjugates by mammalian digestive enzymes (cholesterol esterase and pancreatin, a mixture of pancreatic enzymes) and for comparison purposes, by KOH. Two types of purified hydroxycinnamate esters (sitostanyl ferulate and oryzanol, a mixture of hydroxycinnamate esters purified from rice bran oil) were hydrolyzed by cholesterol esterase and by pancreatin. Both cholesterol esterase and pancreatin hydrolyzed the phytosteryl esters in two functional food matrices, and they hydrolyzed the hydroxycinnamate esters in corn fiber oil. This is the first report to demonstrate that phytostanyl ferulate esters (which are present at levels of 3–6% in corn fiber oil) are hydrolyzed by pancreatic cholesterol esterase. It is also the first report that pancreatin contains enzymes that hydrolyze the fatty acyl moiety of fatty acylated steryl glycoside, converting it to steryl glycoside. Pancreatin had no effect on steryl glycosides. The ability of pancreatin to hydrolyze three other types of lipid conjugates was also evaluated. Phospholipids were completely hydrolyzed. About half of the galactolipids were hydrolyzed, and less than 10% of the polyamine conjugates were hydrolyzed. The extents of hydrolysis of phytosteryl esters by base (saponification) were also studied, and conditions commonly used for the saponification of acyl lipids (1.5 N methanolic KOH, 30 min at 70°C), were found to result in a nearly 100% hydrolysis of TAG but only about 35–45% hydrolysis of the phytosteryl fatty acyl esters or phytosteryl hydroxycinnamate esters.

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In recent years there has been much interest in phytosterols (plant sterols), mainly due to their ability to lower LDL-cholesterol by 10–15%, when consumed at dosages of 1–3 g/d and at still lower dosages in certain food matrices (1). In plants and in plant-derived foods, phytosterols can occur either in the "free" form (FS, with an OH group that is not bound), or as phytosterol conjugates: phytosterol fatty acyl esters (SE), hydroxycinnamate phytosterol esters (HSE), acylated steryl glycosides (ASG), and steryl glycosides (SG) (Fig. 1). FS, SE, ASG, and SG are ubiquitous in plants, but HSE are usually found only in cereals such as corn and rice. Very little is known about how phytosterol conjugates are hydrolyzed and metabolized during digestion. Two *in vivo* studies have demonstrated that SE are hydrolyzed in the upper portion of the small intestine (2,3). One *in vitro* study reported comparable rates of hydrolysis (23–34%)
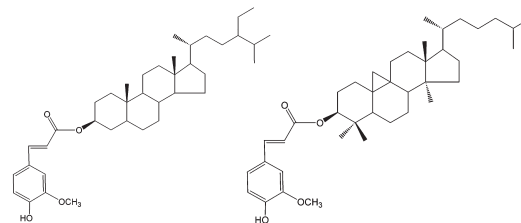
A. Phytosterol fatty acyl esters (SE)

(examples, sitosteryl oleate and sitostanyl oleate)
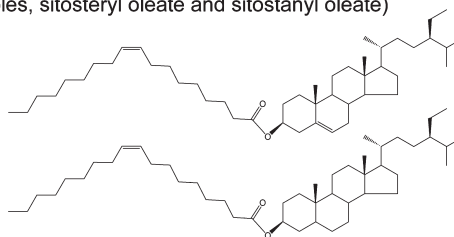
B. Phytosterol hydroxycinnamate esters (HSE)

(examples, sitostanyl ferulate and cycloartenyl ferulate)

C. Phytosterol glycosides

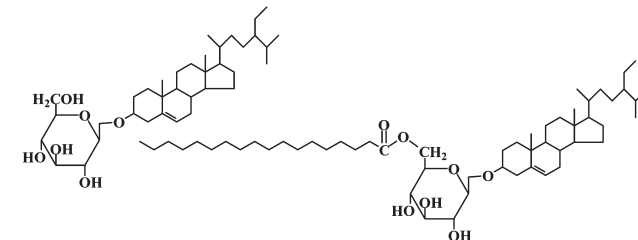(examples, steryl glycoside, SG, and acylated steryl glycoside, ASG)

FIG. 1. Structures of some common phytosterol conjugates found in foods.

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Abbreviations: ASG, acylated steryl glycosides; CFP, *p*-coumaroyl feruloylputrescine; DFP, diferuloylputrescine; FS, free phytosterol, i.e., with an unbound –OH group; HSE, hydroxycinnamate phytosterol esters; SE, phytosterol fatty acyl esters; SG, steryl glycosides.

of various oleate esters of sterols (cholesterol, sitosterol, and stigmasterol) using porcine pancreatic cholesterol esterase (4).

One *in vivo* study demonstrated that oryzanol (an HSE from rice bran oil) is hydrolyzed in the small intestine (5). Two recent *in vitro* studies demonstrated that some HSE (orzyanols comprising desmethyl phytosterols) were rapidly hydrolyzed by pancreatic enzymes whereas others (oryzanol, mainly comprising dimethyl phytosterols such as cycloartenol) were apparently hydrolyzed at much lower rates (6,7).

We are not aware of any previous *in vitro* reports of the ability of digestive enzymes to hydrolyze steryl glycosides or phytostanyl ferulate esters, which are found at levels of 3–6% in corn fiber oil (1). Some previous reports have provided evidence that certain plant β-glucosidases could hydrolyze phytosterol glycosides (8–12) whereas others (1,13), using more highly purified preparations of β-glucosidase, have reported that these enzyme preparations were not able to hydrolyze SG.

This study was undertaken to investigate the extents of *in vitro* hydrolysis of several phytosterol conjugates (especially the phytostanyl ferulate esters in corn fiber oil and steryl glycosides), and other lipid conjugates (Fig. 2), using two commercial mammalian digestive enzymes (bovine cholesterol esterase and pancreatin, of which the latter is a common commercial extract of the pancreas of hogs, containing cholesterol esterase, lipase, amylase, trypsin, and other enzymes) and to compare these enzymatic hydrolyses to those obtained *via* saponification (alkaline hydrolysis).

A. Phospholipids
(example, phosphatidylcholine, PC)

B. Galactolipids
(example, digalactosyldiacylglycerol, DGDG)

C. Polyamine conjugates
(examples, diferuloylputrescine, DFP, and *p*-coumaroyl-feruloylputrescine, CFP

FIG. 2. The structures of some other common plant lipid conjugates found in foods.

MATERIALS AND METHODS

Oryzanol was purchased from CTS Organics (Atlanta, GA). Sitostanyl ferulate (~99%) was synthesized by the method of Condo *et al.* (14). Take Control® and Benecol® were purchased at a local supermarket. Lecithin Granules, 97% Soy Phosphatides (Vitamin Shoppe, North Bergen, NJ) were obtained from a local vitamin store. Oat oil was purchased from BioSeparations (Los Angeles, CA). Corn fiber oil was prepared as previously described (15). An extract rich in polyamine conjugates was prepared as previously described (16). All other reagents were purchased from Sigma (St. Louis, MO).
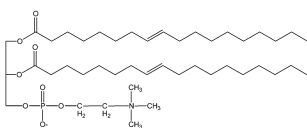
Lipids were added to the reaction mixture first by pipeting a stock solution containing lipids dissolved in isopropanol. Oryzanol and sitostanyl ferulate stock solutions were prepared by dissolving the pure compounds in isopropanol at a concentration of 0.005 M. Benecol, Take Control, and corn fiber oil stock solutions were prepared at a concentration of 40 mg/mL. Lecithin, oat oil, and corn bran ethanol extract stock solutions were prepared at a concentration of 10 mg/mL. Isopropanol was evaporated with a stream of nitrogen, and then the other reagents were added. All enzymatic reactions (5 mL) contained 0.05 M Trizma buffer and pH 7.0, 0.020 taurocholate. Both enzymes (see next) were dissolved in the above buffer at a concentration of 10 mg/mL. Pancreatin (2 mg, from porcine pancreas, Sigma P-7545) and cholesterol esterase (1 mg, from bovine pancreas, Sigma C-3766) were added to each 5-mL reaction mixture. Lipids were extracted by the method of Bligh and Dyer (17).

Nonpolar lipids (including SE, HSE, TAG, and FFA) were quantitatively analyzed by a normal-phase HPLC method with ELSD (15). These analyses were performed on a Model 1050 Hewlett-Packard HPLC, with autosampler, and detection was by both Model 1050 HP diode-array UV-vis detector (Agilent Technologies, Avondale, PA) and an MKII Alltech-Varex Evaporative Light Scattering Detector (Alltech Associates, Deerfield, IL), operated at 40°C and a nitrogen gas flow rate of 1.7 standard liters per minute. The column was a LiChrosorb 7 μ DIOL column (3 × 100 mm, packed by Chrompack, Raritan, NJ). The ternary gradient had a constant flow rate of 0.5 mL/min, with Solvent A = hexane/acetic acid, 1000:1, Solvent B = hexane/isopropanol, 100:1. The gradient timetable was as follows: at 0 min, 100:0 (%A/%B); at 8 min, 100:0; at 10 min, 75:25; at 40 min, 75:25; at 41 min, 100:0; and at 60 min, 100:0.

Polar lipids (including ASG, SG, glycolipids, phospholipids, and polyamine conjugates) were quantitatively analyzed by a similar HPLC-ELSD method (18), using a Model 1100 Hewlett-Packard HPLC, with autosampler, and detection by both a Model 1100 HP diode-array UV-vis detector (Agilent Technologies) and a Model 55 Sedex Evaporative Light Scattering Detector (Richard Scientific, Novato, CA), operated at 40°C and a nitrogen gas pressure of 2 bar. The diol column and flow rates were the same as above. The ternary gradient consisted of Solvent A = hexane/acetic acid, 1000:1; Solvent B = isopropanol; and Solvent C = water. Gradient timetable was as
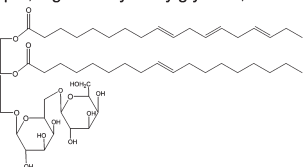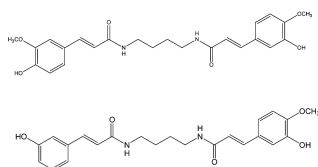
TABLE 1
Hydrolysis of Nonpolar Phytosterol Conjugates (in purified samples) by Digestive Enzymes

Phytosterol conjugate	Enzyme	% Hydrolysis[a]	
		1 h	4 h
Sitostanyl ferulate	Cholesterol esterase	55.4 ± 3.9	84.7 ± 3.8
	Pancreatin	ND[b]	47.3 ± 8.3
Oryzanol	Cholesterol esterase	33.7 ± 4.3	56.3 ± 7.3
	Pancreatin	ND	0

[a]All reported in all tables are the mean ± SD ($n = 3$).
[b]Not determined.

follows: at 0 min, 90:10:0 (%A/%B/%C); at 30 min, 58:40:2; at 40 min, 45:50:5; at 50 min 45:50:5; at 51 min, 50:50:0; at 52 min, 90:10:0; and at 60 min 90:10:0. The minimum limit of quantitative detection with both HPLC methods was about 1 μg per injection. Mass vs. peak area calibration curves were constructed for the range of 1–20 μg per injection.

For enzymatic and saponification studies the extent of hydrolysis was calculated as the decrease in the levels of substrate (phytosteryl conjugates or other lipids). All enzymatic treatments were performed three times, twice with duplicate samples and once with triplicate samples. The enzymatic results presented are the means of the triplicate sample experiment ± SD. The saponification experiments were performed twice, each time with duplicate samples, and the data presented are the means from one experiment.

RESULTS AND DISCUSSION

The first set of experiments focused on evaluating the extent of enzymatic hydrolysis of phytosteryl and phytostanyl fatty acyl esters in two purified phytosteryl conjugates (Table 1). Our *in vitro* studies demonstrated that cholesterol esterase catalyzed a moderate extent of hydrolysis of both HSE, about 55–85% hydrolysis of sitostanyl ferulate (a desmethyl stanyl ester from corn) (Fig. 3), and about 35–55% hydrolysis of oryzanol (a steryl ferulate ester mixture comprising both dimethyl and desmethyl sterols from rice bran). Pancreatin catalyzed the hydrolysis of about half of the sitostanyl ferulate

in 4 h, but none of the oryzanol (Table 1). We do not know why cholesterol esterase catalyzed the hydrolysis of both HSE (oryzanol and sitostanyl ferulate), whereas pancreatin (which contains cholesterol esterase) only hydrolyzed sitostanyl ferulate and not oryzanol. A possible explanation is that the cholesterol esterase was bovine and the pancreatin was porcine, and the amino acid sequences and substrate specificities of the two cholesterol esterases could have been different. Another possible explanation is that some of the other enzymes (lipases, proteases, etc.) or nonenzymatic components in pancreatin, or some of the products generated by some of the other enzymes, may alter the specificity of its cholesterol esterase.

This is the first study of any kind that demonstrates phytostanyl ferulate esters (which are present at levels of 3–6% in corn fiber oil) are hydrolyzed by mammalian digestive enzymes. These *in vitro* data predict that the phytostanyl ferulate esters in corn fiber oil potentially can be hydrolyzed and the free stanols can potentially lower LDL-cholesterol in the same manner as phytosteryl and phytostanyl fatty acyl esters. The efficacy of the phytostanyl and phytosteryl fatty acyl esters has been demonstrated in numerous clinical studies (1).

Huang (6) used reversed-phase HPLC to separate the individual molecular species in oryzanol, before and after incubation with cholesterol esterase, and presented qualitative data (chromatograms) suggesting that the esters with desmethyl phytosterols (sitosteryl and campesteryl ferulates) were hydrolyzed at a much faster rate than the esters of dimethyl phytosterols (cycloartenyl and 24-methylcycloartenyl ferulates). Miller *et al.* (7) performed similar incubations of individual molecular species of oryzanol HSE and demonstrated that desmethyl esters (sitosteryl and campesteryl ferulates) were hydrolyzed by cholesterol esterase and dimethyl esters (cycloartenyl and 24-methylcycloartenyl ferulates) were not. *In situ* experiments indicated that ^{14}C-γ-oryzanol was partially hydrolyzed in the intestine during absorption (5). The lower extents of *in vitro* hydrolysis of esters of dimethyl sterol-containing esters in the current study (oryzanol) and in previous *in vitro* studies may help to explain the lower *in vivo* cholesterol-lowering efficacy of dimethylsterols reported by Trautwein
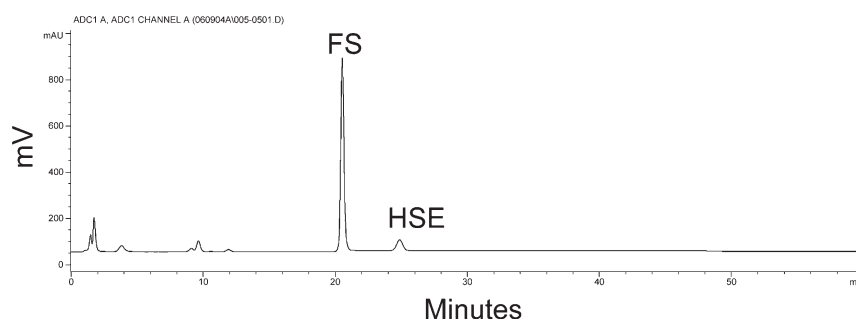

FIG. 3. HPLC chromatogram evaluating the hydrolysis of phytosterol hydroxycinnamate esters. The substrate was pure sitostanol ferulate (0.15 mg), the enzyme was cholesterol esterase (1 mg), in 5 mL reaction mixture or buffer and taurocholate, which was incubated for 1 h at 37°C. Abbreviations: FS, free phytosterols; HSE, sitostanyl ferulate.

TABLE 2
Hydrolysis of Nonpolar Phytosterol Conjugates (in food matrices) by Digestive Enzymes

Food matrix	Phytosterol conjugate	Concentration of Enzyme conjugate in food matrix (wt% of total lipid)		% Hydrolysis	
				1 h	4 h
Benecol®	Phytostanyl FA esters	~10%	Cholesterol esterase[a]	33.0 ± 2.8	44.6 ± 9.6
			Pancreatin	ND	8.0 ± 4.2
Take Control®	Phytosteryl FA esters	~10%	Cholesterol esterase	38.1 ± 2.4	46.0 ± 4.8
			Pancreatin	ND	35.6 ± 2.3
Corn fiber oil	Phytosteryl FA esters	~6%	Cholesterol esterase	9.6 ± 3.1	10.1 ± 5.4
			Pancreatin	ND	0
	Phytostanyl ferulate	~4%	Cholesterol esterase	26.7 ± 4.2	57.0 ± 1.1
			Pancreatin	ND	10.5 ± 4.4

[a]*Note:* With all three food matrices, the substrate also included TAG, and TAG were completely hydrolyzed in 4 h. ND, not determined.

et al. (19). It should be noted that the previous two *in vitro* reports did not include any phytostanyl esters, so our current report is the first *in vitro* report of the hydrolysis of a phytostanyl ferulate ester by mammalian digestive enzymes

Although these experiments demonstrated that *mammalian* cholesterol esterase is able to hydrolyze HSE, it should also be noted that in our previous report (20) the *microbial* cholesterol esterase (in a commercial spectrophotometric cholesterol test kit) was unable to hydrolyze sitostanyl ferulate.

The second experiment investigated the extent of hydrolysis of several phytosteryl ester-containing functional foods (Table 2). The phytosteryl and phytostanyl fatty acyl esters in the two margarines were hydrolyzed to a similar extent at both 1 h (33–38%) and 4 h (44–46%) (Table 2). An interesting observation was that in addition to hydrolyzing the steryl esters, the purified cholesterol esterase catalyzed the hydrolysis of almost all of the TAG in all three food matrices (Fig. 4). When the phytosteryl esters in both spreads were incubated with pancreatin, a slightly lower extent of hydrolysis was observed with the steryl esters in Take Control (about 36% hydrolysis), and a much lower extent of hydrolysis was observed with stanyl esters in Benecol (about 8% hydrolysis). When the cholesterol esterase was used to hydrolyze corn fiber oil, its phytosteryl fatty acyl esters (which constitute 4–6% of the oil) were hydrolyzed to a low extent (9–10%) and its hydroxycinnamate esters [predominantly sitostanyl ferulate according to

Norton (21)] were hydrolyzed to a moderate extent (27–57%). When pancreatin was used to hydrolyze corn fiber oil, its phytostanyl ferulate was hydrolyzed to a low extent (about 11%); there was no apparent hydrolysis of its phytosteryl fatty acyl esters. This lower rate of hydrolysis of the corn fiber oil HSE than the rate of hydrolysis of pure sitostanyl ferulate (Table 1) may be attributed to the fact that the former is a mixture of esters that contains significant levels of campestanol and other esterified phytostanols and phytosterols (21). The phytosteryl fatty acyl esters in corn fiber oil were hydrolyzed to a lesser extent than those in both margarines. A possible explanation for the low extent of hydrolysis of phytosteryl fatty acyl esters in corn fiber oil is that the presence of other components (wax esters, squalene, and other hydrocarbons, which have retention times very close to that of phytosteryl fatty acyl esters), may interfere with the accurate quantification of these esters. Since the extent of hydrolysis was calculated as the disappearance of substrate, the presence of these compounds could contribute to the apparent lower extents of hydrolysis with corn fiber oil. In the future we will test this hypothesis using another HPLC system (reversed-phase or alumina) that better separates these very nonpolar components.

In a previous *in vitro* study, the rates of hydrolysis of several molecular species of phytosteryl fatty acyl esters by porcine pancreatic cholesterol esterase were shown to be similar to the rates of hydrolysis of cholesteryl esters (4). The
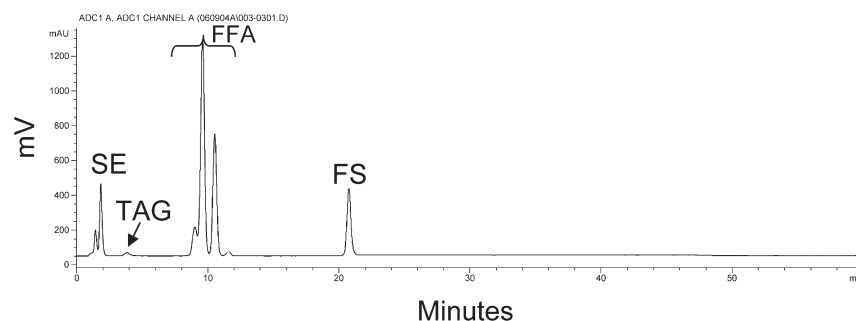

FIG. 4. HPLC chromatogram evaluating the hydrolysis of phytosterol fatty acyl esters (SE). The substrate was Take Control® (2 mg), the enzyme was cholesterol esterase (1 mg), in a 5-mL reaction mixture of buffer and taurocholate, incubated for 1 h at 37°C. Abbreviation: FS, free phytosterols.
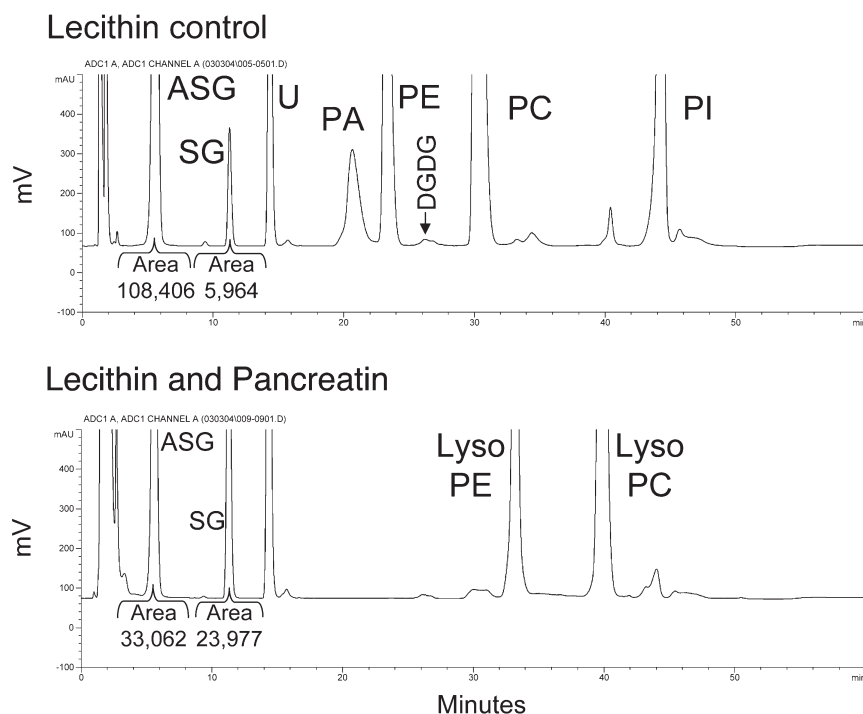
Lecithin control

Lecithin and Pancreatin

FIG. 5. HPLC chromatogram evaluating the hydrolysis of phytosterol glycosides (ASG and SG) and phospholipids. The substrate was soy lecithin (2 mg) and the enzyme was pancreatin (2 mg) in 5 mL of a reaction mixture of buffer and taurocholate that was incubated for 6 h at 37°C. U, unknown; DGDG, digalactosyldiacyglycerol; for other abbreviations see Figure 1.

current and previous *in vitro* results confirmed the *in vivo* reports (2,3) that phytostanyl and phytosteryl fatty acyl esters (SE) are hydrolyzed in the mammalian GI system.

The next several experiments were performed to evaluate the ability of pancreatin to hydrolyze phytosteryl glycosides and other polar lipid conjugates. An HPLC system designed to separate polar lipids was employed (Figs. 5,6) and the results are summarized in Table 3.

Among the steryl glycoside conjugates, pancreatin was found to hydrolyze the FA moiety from ASG, thus converting it to SG (Table 3). However, with three different food matrices (Table 3), the glycosidic bond in SG apparently was not hydrolyzed by pancreatin (Fig. 5). It is not widely known that "Soy Lecithin" or "Lecithin Granules" contain significant levels of sterol glycosides. Also, it is unfortunate that the word lecithin has two meanings: It is the common name for PC, and it is the name for a food supplement that is an enriched fraction of polar lipids, containing about 97% phosphatides. The manufacturer claims that the fraction contains 3% "other lipids;" our analytical data indicate that it contains about 3% phytosterols (~2.7% acylated steryl glycoside and ~0.2% steryl glycoside, Table 3). Oat oil and corn bran ethanol extract were evaluated as other food matrices that also contain SG and that contain other lipid conjugates. Because the levels of SG actually increased when all three food matrices were incubated with pancreatin (presumably owing to the hydrolysis of ASG), we feel that it will be important to confirm this observation (i.e., the inability of the enzyme to hydrolyze

SG) in the future by evaluating the ability of pancreatin to hydrolyze a purified preparation of SG.

Weber (22) performed *in vivo* metabolic studies with rats. His results indicated that [4-^{14}C]sitosteryl β-D-glucosides were hydrolyzed at a low rate in the intestines, but they were not absorbed. In contrast, the current *in vitro* results indicate that SG is not hydrolyzed by mammalian digestive enzymes.

Although this is the first published *in vitro* report evaluating the potential hydrolysis of steryl glycosides by mammalian digestive enzymes, others have reported that some early preparations of β-glucosidase from almonds were useful for the hydrolysis of SG (8–12). However, it has been reported that more recent commercial preparations of almond β-glucosidase were unable to hydrolyze SG (1,13). A possible explanation is that there are multiple types of β-glucosidase in almond, and that the type(s) that hydrolyze SG are not the type(s) that are enriched in modern commercial preparations. Recent evidence suggests that one possible physiological role of steryl glycosides is to serve as primers for cellulose synthesis (23).

Among the other common plant lipid conjugates, pancreatin causes near-complete (>99%) hydrolysis of phospholipids in Lecithin Granules (Fig. 5) and a partial (about 40%) hydrolysis of digalactosydiacylglycerol (Fig. 6), a common plant galactolipid, which is present at a level of 1–5% in oat oil.

We recently reported (16) that the pericarp of corn kernels contains high levels of two unusual polyamine conjugates, diferuloylputrescine (DFP) and *p*-coumaroyl feruloylputrescine (CFP) (structures shown in Fig. 2). When corn bran
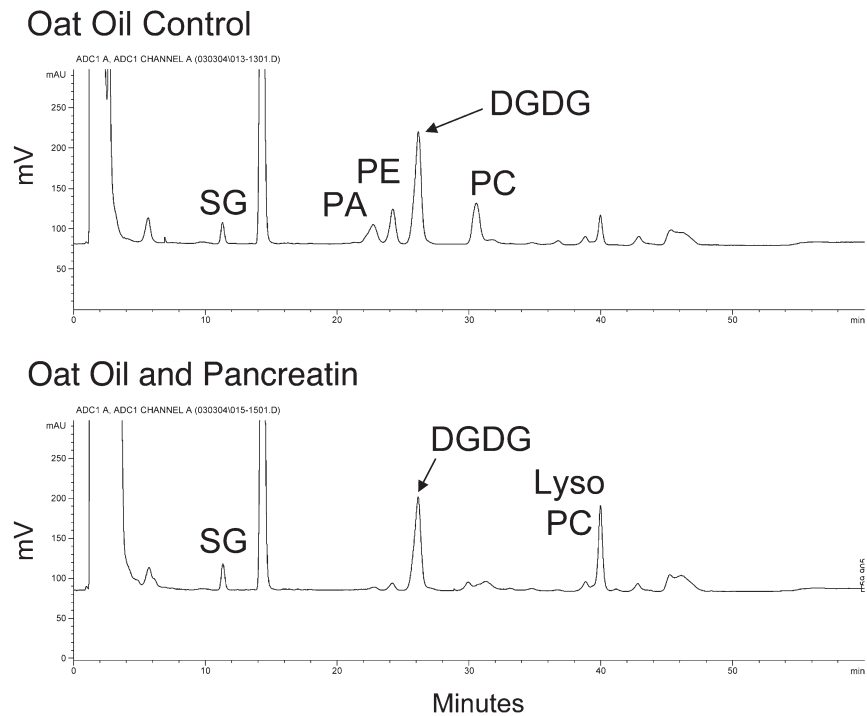
Oat Oil Control

Oat Oil and Pancreatin

FIG. 6. HPLC chromatogram evaluating the hydrolysis of DGDG. The substrate was oat oil (2 mg) and the enzyme was pancreatin (2 mg) in 5 mL of a reaction mixture of buffer and taurocholate that was incubated for 6 h at 37°C. For abbreviations see Figures 1 and 5.

is extracted with hot ethanol, the extract contains about 10% DFP and 2% CFP (16). When the potential hydrolysis of this extract was evaluated, these two conjugates were hydrolyzed to a slight extent (about 2% hydrolysis of DFP and about 8% hydrolysis of CFP) (Table 3). In this HPLC system, the retention times for CFP and DFP were 24 and 25 min, respectively (data not shown).

Saponification (alkaline hydrolysis) of lipid extracts is a routine procedure to hydrolyze the ester bonds before analyzing the resulting FA or free phytosterols (Table 4). Christie

(24) cautioned that saponification of steryl esters requires longer and more vigorous saponification than saponification of common acylglycerol esters. Thompson and Merola (25) reported that cholesteryl oleate was completely hydrolyzed in 8 min upon saponification with 0.5 N KOH in ethanol/pyrogallol (97:3) at 80°C. In the current study, 30 min was adequate for the complete (>99%) hydrolysis of the TAG in the two margarines, but it resulted in only about 35–65% hydrolysis of the four phytosterol ester conjugates (Table 4). Saponification for 2 h resulted in about 96 and 86% hydrolysis of the

TABLE 3
Hydrolysis of Phytosterol Conjugates (glycosides) and Other Polar Lipids (in food matrices) by Pancreatin

Food matrix	Phytosterol conjugate (or other lipid)	Concentration of conjugate in food matrix	% Hydrolysis at 6 h
Lecithin granules	Acylated steryl glycoside, ASG	~2.7%	54.3 ± 0.6
	Steryl glycoside, SG	~0.2%	0 (380% increase)
	Phospholipids, PC	~97%	>99
Oat oil	Acylated steryl glycoside, ASG	~0.1	NQ[a]
	Steryl glycoside, SG	~0.2%	0 (22% increase)
	Galactolipid, DGDG	~3%	39.7 ± 0.6
	Phospholipids, PC	~2%	>99
Corn bran ethanol extract	Acylated steryl glycoside, ASG	~0.1%	NQ
	Steryl glycoside, SG	~0.1%	0 (80 % increase)[a]
	Polyamines, DFP	~5%	1.8 ± 1.6
	CFP	~1%	7.8 ± 4.1
	Phospholipids, PC	~2%	>99

[a]NQ, not quantified. In oat oil and corn bran ethanol extract, the ASG peak was masked by other peaks and could not be accurately estimated. However, the increase in SG is evidence that ASG was present and it was hydrolyzed. DGDG, digalactosyldiacylglycerol; DFP, diferuloylputrescine; CFP, p-coumaroyl feruloylputrescine.

TABLE 4
Hydrolysis of Phytosterol Conjugates (esters) by Saponification with 1.5 N KOH in Methanol (10% water) at 70°C

Food matrix or pure compound	Phytosterol conjugate	% Hydrolysis at 30 min	% Hydrolysis at 120 min
Benecol®	Phytostanyl fatty acyl esters	52.2[a]	96.7
Take Control®	Phytosteryl fatty acyl esters	64.4[a]	97.4
Purified sample	Sitostanyl ferulate	34.7	87.0
Purified sample	Oryzanol	44.6	86.2

[a]*Note:* TAG in these samples were completely hydrolyzed within 30 min.

SE and HSE, respectively. Our results differ from those of Thomson and Merola (25) and indicate that any analyses that include alkaline hydrolysis of sterols should include methods to verify complete hydrolysis.

This report provides the first *in vitro* evidence that phytostanyl ferulate esters (a major component in corn fiber oil) are hydrolyzed by mammalian digestive enzymes. It is also the first report that provides evidence that ASG are hydrolyzed to steryl glycosides by mammalian digestive enzymes. However, the glycosidic linkage in steryl glycosides does not appear to be hydrolyzed by mammalian digestive enzymes. This study also reports the first *in vitro* data demonstrating the extent of hydrolysis of phytostanyl fatty acyl esters in food matrices by mammalian digestive enzymes. Because all plant-derived foods, including most functional foods, and some supplements (e.g., Lecithin Granules) contain steryl glycosides and most grains contain phytostanyl ferulate esters, these new findings have implications in the fields of food science, nutraceuticals, and pharmacology. It also confirms several previous reports of the *in vitro* hydrolysis of some phytosterol conjugates (phytosteryl fatty acyl esters, oryzanol, and other phytosteryl-ferulates). Our results indicate the bovine preparation of cholesterol esterase could be useful for those interested in using it as a tool to hydrolyze phytosteryl esters. On the other hand, the porcine pancreatin preparation used in these studies provided reasonable and repeatable hydrolysis of ASG to SG (and hydrolysis of phospholipids and galactolipids) but it appears to be less useful as a tool for the hydrolysis of the phytosteryl esters.

REFERENCES

1. Moreau, R.A., Whitaker, B.D., and Hicks, K.B. (2002) Phytosterols, Phytostanols, and Their Conjugates in Foods: Structural Diversity, Quantitative Analysis, and Health-Promoting Uses, *Prog. Lipid Res. 41,* 457–500.
2. Miettinen, T.A., Vuoristo, M., Nissinen, M., Jarvinen, H.J., and Gylling, J. (2000) Serum, Biliary and Fecal Cholesterol and Plant Sterols in Colectomized Patients Before and During Consumption of Stanol Ester Margarine, *Am. J. Clin. Nutr. 71,* 1095–1102.
3. de Jong, A., Plat, J., and Mensink, R.P. (2003) Metabolic Effects of Plant Sterols and Stanols, *J. Nutr. Biochem. 14,* 262–269.
4. Swell, L., Field, H., Jr., and Treadwell, C.R. (1954) Sterol Specificity of Pancreatic Cholesterol Esterase, *Proc. Soc. Exptl. Biol. Med. 87,* 216–218.
5. Fujiwara, S., Sakurai, S., Noumi, Sugimoto, I., and Awata, N. (1983) Absorption and Metabolism of γ-Oryzanol in Rats, *Chem. Pharm. Bull. 31,* 645–652.
6. Huang, C-C.J. (2003) Potential Functionality and Digestibility of Oryzanol as Determined Using *in vitro* Cell Culture Methods, Ph.D. Dissertation, Louisiana State University, pp. 119–139. Also available at netlink: http://etd02.lnx390.lsu.edu/docs/available/etd-0609103-135757/.
7. Miller, A., Majauskaite, L., and Engel, K-H. (2004) Enzyme-Catalyzed Hydrolysis of γ-Oryzanol, *Eur. Food Res. Technol. 218,* 349–354.
8. Kesselmeier, J., Eichenberger, W., and Urban, B. (1985) High-Performance Liquid Chromatography of Molecular Species of Free Sterols and Sterylglycosides Isolated from Oat Leaves and Seeds, *Plant Cell. Physiol. 26,* 463–471.
10. Moreau, R.A., Powell, M.J., Whitaker, B.D., Bailey, B.A., and Anderson, J.D. (1994) Xylanase Treatment of Plant Cells Induces Glycosylation and Fatty Acylation of Phytosterols, *Physiol. Plant. 91,* 575–580.
11. Palta, J., Whitaker, B.D., and Weiss, L. (1993) Plasma Membrane Lipids Associated with Genetic Variability in Freezing Tolerance and Cold Acclimation of *Solanum* Species, *Plant Physiol. 103,* 793–803.
12. Whitaker, B.D., Lee, E.H., and Rowland, R.A. (1990) EDU and Ozone Protection: Foliar Glycerolipids and Steryl Lipids in Snapbean Exposed to O$_3$, *Physiol. Plant. 80,* 286–293.
13. Holkeri, L.I. (2002) Enzymatic Hydrolysis of Steryl Conjugates II. Analysis of Sterols in Rye, M.Sc. Thesis, University of Helsinki, pp. 25–27.
14. Condo, A.M., Baker, D.C., Moreau, R.A., and Hicks, K.B. (2001) Improved Method for the Synthesis of *trans*-Feruloyl-β-sitostanol, *J. Agric. Food Chem. 49,* 4961–4964.
15. Moreau, R.A., Powell, M.J., and Hicks, K.B. (1996) Extraction and Quantitative Analysis of Oil from Commercial Corn Fiber, *Ibid. 44,* 2149–2154.
16. Moreau, R.A., Nuñez, A., and Singh, V. (2001) Diferuloylputrescine and *p*-Coumaroyl Feruloylputrescine, Abundant Polyamine Conjugates in Lipid Extracts of Maize Kernels, *Lipids 36,* 839–844.
17. Bligh, E.G., and Dyer, W.J. (1959) Rapid Method of Total Lipid Extraction and Purification, *Can. J. Biochem. Physiol. 37,* 911–917.
18. Moreau, R.A., Powell, M.J., and Singh, V. (2003) Pressurized Liquid Extraction of Polar and Nonpolar Lipids in Corn and Oats with Hexane, Methylene Chloride, Isopropanol, and Ethanol, *J. Am. Oil Chem. Soc. 80,* 1063–1067.
19. Trautwein, E.A., Schulz, C., Rieckhoff, D., Kunath-Rau, A., Erbersdobler, H.F., de Groot, W.A., and Meijer, G.W. (2002) Effect of Esterified 4-Desmethylsterols and -Stanols or 4,4'-Dimethylsterols on Cholesterol and Bile Acid Metabolism in Hamsters, *Br. J. Nutr. 87,* 227–237.
20. Moreau, R.A., Powell, M.J., and Hicks, K.B. (2003) Evaluation of a Commercial Enzyme-Based Serum Cholesterol Test Kit

for the Analysis of Phytosterol and Phytostanol Products, *J. Agric. Food Chem. 51,* 6663–6667.

21. Norton, R.A. (1995) Quantitation of Steryl Ferulate and *p*-Coumarate Esters from Corn and Rice, *Lipids 30,* 269–274.

22. Weber, N. (1988) Metabolism of Sitosteryl-β-D-glucoside and Its Nutritional Effects in Rats, *Lipids 23*, 42–47.

23. Peng, L., Kawagoe, Y., Hogan, P., and Delmer, D. (2002) Sitosterol-β-glucoside as Primer for Cellulose Synthase in Plants, *Science 295,* 147–150.

24. Christie, W.W. (2003) *Lipid Analysis: Isolation, Separation, Identification and Structural Analysis of Lipids,* 3rd edn., p. 206, The Oily Press, Bridgewater, United Kingdom.

25. Thompson, R.H., and Merola, G.V. (1993) A Simplified Alternative to the AOAC Official Method for Cholesterol in Multi-Component Foods, *JAOAC 76*, 1057–1068.